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CD40, but Not CD40L, Is Required for the Optimal Priming of T Cells and Control of Aerosol *M. tuberculosis* Infection

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Summary

CD40$^{-/-}$ mice succumbed to low-dose aerosol infection with *M. tuberculosis* due to deficient IL-12 production leading to impaired priming of IFN-γ T cell responses. In contrast, CD40L$^{-/-}$ mice were resistant to *M. tuberculosis*. This asymmetry in outcome of infection between the two knockout strains is likely due to the existence of an alternative ligand for CD40. Both in vitro *M. tuberculosis* infection and recombinant *M. tuberculosis* Hsp70 elicited IL-12 production from WT dendritic cells. This response was absent in both CD40$^{-/-}$ dendritic cells and CD40$^{-/-}$ mice, suggesting that *M. tuberculosis* Hsp70 serves as an alternative ligand for CD40 in vivo.

Introduction

Studies in CD40$^{-/-}$ and CD40L$^{-/-}$ mice demonstrated a critical role for CD40/CD40L interaction in the protective immune response against intracellular parasitic pathogens such as *Leishmania spp* and *Trypanosoma cruzi* (Campbell et al., 1996; Chaussabel et al., 1999; Kamanaka et al., 1996; Soong et al., 1996). In these models, CD40$^{-/-}$ and CD40L$^{-/-}$ mice were unable to control the growth of parasites and succumbed to infection due to impaired IFN-γ production by T cells.

However, CD40/CD40L interaction is not required to mount a protective immune response against all intracellular pathogens. Strong activation of primary CD8 T cell responses after infection of CD40L$^{-/-}$ mice with *Listeria monocytogenes* or certain viruses suggests that priming of CD8 T cells can occur independently of CD40/CD40L interaction (Borrow et al., 1996; Grewal et al., 1997; Thomsen et al., 1998; Whitmire et al., 1996). The induction of CD40/CD40L-independent immune responses against some viruses can be explained by the observation that infection of APC with certain pathogens is sufficient to activate APC thus bypassing the need for costimulation (Ridge et al., 1998).

We and others have previously demonstrated that control of *Mycobacterium tuberculosis* acute and chronic infection is dependent on CD4 T cells (Caruso et al., 1999; Mogues et al., 2001; Saunders et al., 2002; Scanga et al., 2000). Although CD4 T cells are clearly important for IFN-γ production and macrophage activation, it appears that these cells have additional roles in control of *M. tuberculosis* infection (Scanga et al., 2000). Since CD4 T cells are the primary source of CD40L, we questioned whether CD40L interaction with CD40 on APCs was an important function of CD4 T cells in *M. tuberculosis* infection. Our initial hypothesis was that this interaction is not essential for generation of protective immune responses for several reasons. (1) Infection of both human and murine DCs with *M. tuberculosis* was sufficient to upregulate cell surface expression of antigen-presenting and costimulatory molecules as well as induce IL-12 production (Bodnar et al., 2001; Henderson et al., 1997). (2) CD8 T cells were primed to produce IFN-γ in the absence of CD4 T cells in vitro and in *M. tuberculosis*-infected mice (Caruso et al., 1999; Serbina et al., 2001). (3) CD40L$^{-/-}$ mice were resistant to intravenous infection with *M. tuberculosis* (Campos-Neto et al., 1998).

Contrary to our expectations, CD40$^{-/-}$ mice were susceptible to aerosol infection with *M. tuberculosis* due to poor priming of IFN-γ-producing T cells in the lymph nodes as a result of attenuated IL-12 production. The subsequent deficiency in IFN-γ-producing T cells in the lungs of CD40$^{-/-}$ mice culminated in fatal, uncontrolled bacterial growth. However, CD40L$^{-/-}$ mice were resistant to aerosol infection, confirming a previously published study (Campos-Neto et al., 1998). Such asymmetry between the outcome of infection in CD40$^{-/-}$ and CD40L$^{-/-}$ mice points to the existence of an additional ligand for CD40. Here, we show that *M. tuberculosis* Hsp70 functions as an alternative ligand for CD40 as it induced significant IL-12 production by WT but not CD40$^{-/-}$ dendritic cells. Overall, our results highlight an important role for ligation of the CD40 molecule on APC in the control of *M. tuberculosis* infection.

Results

CD40$^{-/-}$ Mice Are Susceptible to Aerosol *M. tuberculosis* Infection

The course of infection was compared in CD40$^{-/-}$ and WT mice after low-dose aerosol infection with virulent *M. tuberculosis* (20–50 cfu/mouse). Bacterial numbers were similar for the first 2 weeks postinfection between the two groups of mice. As colony forming units (cfu) began to plateau in the WT mice at 3 weeks postinfection, bacterial numbers continued to rise in the lungs and spleen of the CD40$^{-/-}$ mice reaching 500- to 1000-fold higher bacterial burden by 4 weeks postinfection (Figures 1A and 1B). Indeed, 40% of the CD40$^{-/-}$ mice were moribund between 3 and 4 weeks postinfection. CD40$^{-/-}$ mice that survived this crisis point had reduced bacterial numbers by 5 weeks postinfection. A small number of surviving CD40$^{-/-}$ mice were followed up to 5 months postinfection; these mice had excessive lung pathology and 10- to 1000-fold higher cfu in the lungs compared to WT mice.

Histological analysis did not reveal obvious differ-

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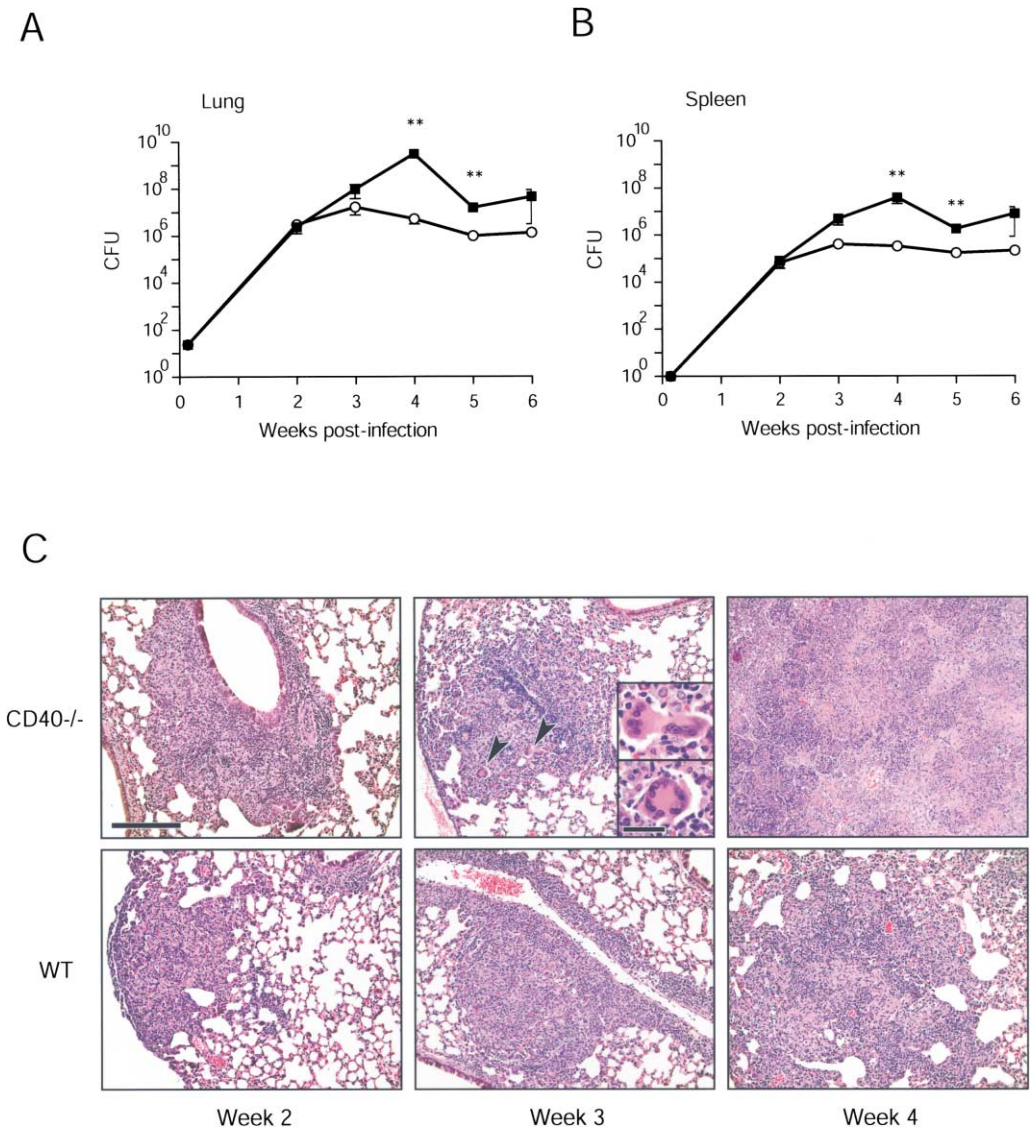

Figure 1. CD40$^{-/-}$ Mice Are Susceptible to Aerosol *M. tuberculosis* Infection

(A and B) CD40$^{-/-}$ (■) and C57BL/6 (○) mice were infected with ~20–50 cfu of *M. tuberculosis* via aerosol. At weekly time points the number of cfu was determined by plating serial dilutions of the lung (A) and spleen (B) homogenates.

(C) At 2, 3, and 4 weeks postinfection lung sections of CD40$^{-/-}$ and WT mice were stained with hematoxylin and eosin. Scale bar equals 200 μm, inset scale bar is 30 μm. The data are representative of three experiments, with four mice per experimental group at each time point.

ences in the infiltration of cells into the lungs in the first 3 weeks of infection (Figure 1C). Analysis of lung sections from CD40$^{-/-}$ mice revealed the presence of unusual, multinucleated giant cells at 3 weeks postinfection (Figure 1C). These giant cells are the hallmark of human tuberculosis but are rarely found in mice except in chronic infection as a result of long-term inflammation (data not shown).

CD40 Ligation Is Not Required for the Induction of Mycobactericidal Mechanisms in Macrophages

Production of reactive nitrogen intermediates (RNI) via the inducible nitric oxide synthase (NOS2) pathway by activated macrophages is an important component of macrophage-mediated defense against *M. tuberculosis* (Chan et al., 1992; MacMicking et al., 1997). CD40/CD40L interaction was critical for T cell-dependent activation of macrophages resulting in RNI production and microbicidal activity against *Leishmania* and other intracellular pathogens (Campbell et al., 1996; Chaussabel et al., 1999; Kamanaka et al., 1996; Soong et al., 1996). To address the role of CD40/CD40L interaction in macrophage activation, in vivo expression of CD40 following *M. tuberculosis* infection was examined. By 1 week postinfection, only 5% of lung cells within the R1 gate expressed CD40, but this number increased to 50% by 4 weeks postinfection (Figure 2A).

To test the dependence of macrophage effector functions on CD40 ligation, the ability of CD40$^{-/-}$ and WT macrophages to limit the growth of intracellular mycobacteria and produce nitrite (a measure of RNI) was evaluated in vitro. CD40$^{-/-}$ and WT macrophages were

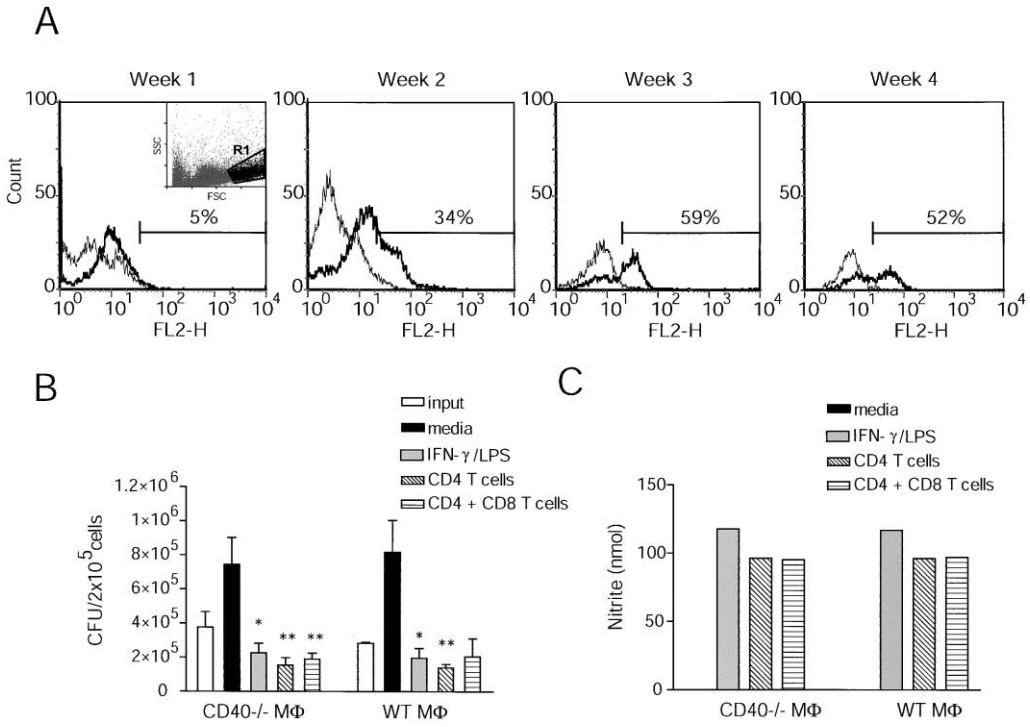

Figure 2. CD40 Ligation Is Not Required for the Induction of a Mycobactericidal State in Macrophages

(A) Expression of CD40 on lung cells during the course of aerosol *M. tuberculosis* infection was determined by flow cytometry. Lung cells from WT mice were stained with anti-CD40 antibody (thick line) or isotype control (thin line), and the percentage of CD40 positive cells within R1 gate was determined.

(B) The ability of CD40$^{-/-}$ and WT Mϕ to reduce the number of intracellular bacteria was assessed by an in vitro macrophage killing assay as described in the Experimental Procedures. Error bars are standard error of mean, and the p values were calculated by comparing each condition to the input cfu. *p value $\leq$ 0.05; **p value $\leq$ 0.01.

(C) The amount of nitrite in supernatants of resting Mϕ (media alone) or IFN-γ/LPS and T cell-activated Mϕ was determined by a Greiss assay. The data are representative of three experiments (B and C) with four mice per experimental group at each time point.

infected with *M. tuberculosis* (Figure 2B, input) and cultured for 3 days in media alone, IFN-γ/LPS, or with unfractionated or CD8-depleted lung T cells isolated from 4-week-infected WT mice. CD40$^{-/-}$ and WT macrophages were equally efficient at reducing the numbers of intracellular mycobacteria after IFN-γ/LPS or T cell activation (Figure 2B). Furthermore, similar amounts of nitrite were produced when infected CD40$^{-/-}$ and WT macrophages were activated with IFN-γ/LPS or mycobacteria-specific T cells (Figure 2C). No reduction in the number of intracellular mycobacteria or nitrite production was observed when macrophages were cultured with naive splenic T cells (data not shown). These results indicate that CD40$^{-/-}$ macrophages did not differ significantly from WT macrophages in their intrinsic ability to produce RNI or limit the growth of intracellular bacteria. Hence, CD40/CD40L interaction is not pivotal to the induction of NOS2 expression in *M. tuberculosis*-infected mice.

CD40$^{-/-}$ DCs Are Inefficient in Priming Naive T Cells Despite Normal Maturation and Migration into the Lung-Draining Lymph Nodes

CD40/CD40L interaction has an important role in the maturation of APC, characterized by upregulation of costimulatory and antigen-presenting molecules and production of inflammatory cytokines (Cella et al., 1996; Grewal et al., 1996). To ascertain the role of CD40/CD40L interaction in DC activation, cell surface molecule expression as a result of *M. tuberculosis* infection and CD40 ligation was examined. Infection of both WT and CD40$^{-/-}$ DCs with *M. tuberculosis* resulted in upregulation of MHC class I, MHC class II, and B7.2, suggesting that *M. tuberculosis* infection alone was sufficient to mature CD40$^{-/-}$ and WT DCs (data not shown). In vivo, migration of DCs into the lung-draining lymph nodes of WT and CD40$^{-/-}$ mice after aerosol challenge was comparable (Figure 3A). These results indicate that CD40$^{-/-}$ and WT DCs are equally equipped with antigen-presenting and costimulatory machinery and exhibit no defect in their in vivo migratory abilities.

Next, we investigated whether CD40/CD40L interaction resulted in improved induction of *M. tuberculosis*-specific T cell responses. Naive splenocytes were primed with *M. tuberculosis*-infected WT or CD40$^{-/-}$ DCs for 7 days in vitro. The frequency of *M. tuberculosis*-specific IFN-γ-producing T cells after priming with infected WT DCs was 2-fold higher than in T cell cultures that were primed with infected CD40$^{-/-}$ DCs (Figure 3B).

The most striking difference between WT and CD40$^{-/-}$ DCs was observed in their capacity to produce IL-12 following *M. tuberculosis* infection. Incubation with stimulating anti-CD40 antibody or infection with *M. tuberculosis* in the absence of T cells induced a 4-fold increase

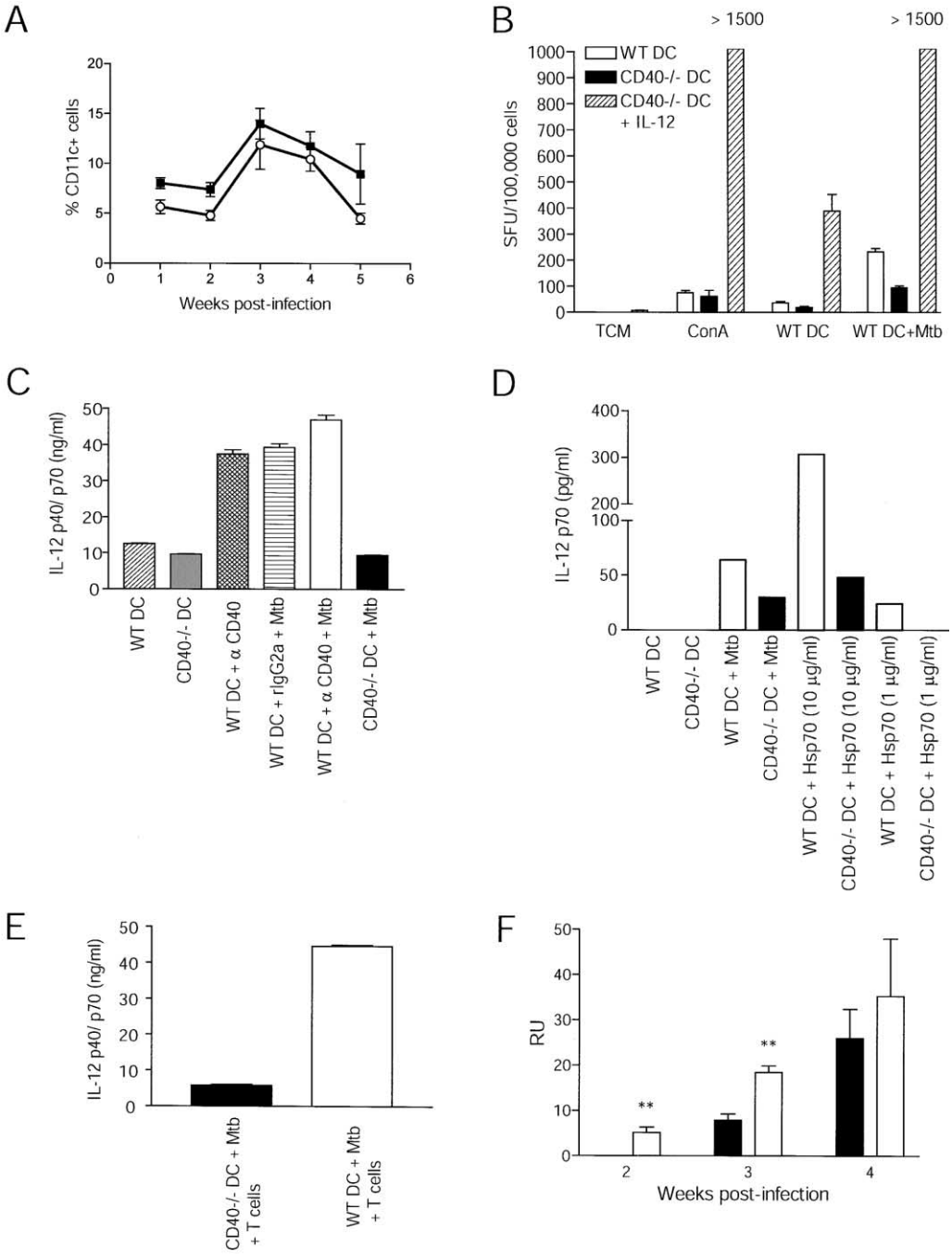

Figure 3. CD40$^{-/-}$ DCs Are Inefficient at Priming Naive T Cells and Show Diminished IL-12 Production In Vitro and In Vivo

(A) Single-cell suspensions from lymph nodes of CD40$^{-/-}$ (■) and WT (○) mice were stained with anti-CD11c antibodies. The percentage of CD11c^{+} cells within the R1 gate is shown.

(B) The ability of CD40$^{-/-}$ and WT DCs to prime naive T cells was assessed by in vitro priming assay as described in the Experimental Procedures.

(C) The amount of IL-12 produced by uninfected or *M. tuberculosis*-infected CD40$^{-/-}$ DCs and uninfected and *M. tuberculosis*-infected WT DCs treated with either stimulating anti-CD40 antibody or isotype control was determined by ELISA.

(D) WT and CD40$^{-/-}$ DCs were cultured in media alone, infected with *M. tuberculosis* (MOI 6), or treated with 10 μg/ml or 1 μg/ml of *M. tuberculosis*-derived Hsp70 for 24 hr. The amount of IL-12 in the supernatants was determined by ELISA.

(E) IL-12 levels in WT DC-T cell or CD40$^{-/-}$ DC-T cell cocultures during the in vitro priming experiment were quantified by ELISA. The data are representative of three (B and C) or two (E) experiments.

(F) The amount of IL-12 mRNA in the lymph nodes of CD40$^{-/-}$ (■) and WT (□) mice was determined by quantitative RT-PCR as described in the Experimental Procedures.

in IL-12 production by WT DCs (Figure 3C). In contrast, *M. tuberculosis* infection failed to trigger IL-12 production in CD40$^{-/-}$ DCs above baseline, suggesting that IL-12 production after *M. tuberculosis* infection was CD40 dependent (Figure 3C). As Wang et al. reported that CD40 is a receptor mediating mycobacterial heat shock protein 70 stimulation of CC-chemokines (Wang et al., 2001, 2002), we next tested whether recombinant Hsp70 could stimulate IL-12 production through CD40 ligation. Our data indicate that in a T cell-free system, 10 μg/ml of purified LPS-free Hsp70 induced substantial IL-12 production by WT but not CD40$^{-/-}$ dendritic cells (Figure 3D). In addition, the level of IL-12 in the CD40$^{-/-}$ DC-T cell cultures was also 4-fold lower than in the WT DC-T cell cocultures from the in vitro priming experiments (Figure 3E). Consequently, the priming of IFN-γ T cell responses was deficient (Figure 3B). Addition of 50 ng/ml of IL-12 to CD40$^{-/-}$ DC–T cell cultures greatly enhanced the priming of IFN-γ T cell responses (Figure 3B). Most importantly, CD40$^{-/-}$ mice produced significantly less IL-12 in the lymph nodes during the peak of priming at 2 and 3 weeks postinfection (Figure 3F). These results suggest that although infection of DCs with *M. tuberculosis* is sufficient to induce phenotypic changes indicative of maturation, CD40 ligation is essential for optimal IL-12 production by infected DCs in vitro and in vivo and consequently for the priming of mycobacteria-specific, IFN-γ-producing T cells.

Susceptibility of CD40$^{-/-}$ Mice to *M. tuberculosis* Is Associated with Impaired IFN-γ Production

Given that CD40$^{-/-}$ DCs were inefficient at priming naive T cells in vitro, the requirement for CD40 in induction of protective, *M. tuberculosis*-specific Th1 responses in vivo was investigated. CD40$^{-/-}$ mice had substantially fewer CD4 and CD8 T cells in the lungs than WT mice (Figure 4A). The most striking difference was seen at 4 weeks following aerosol challenge when many CD40$^{-/-}$ mice succumbed to infection. This is the time of the peak response in WT mice, after which the T cell numbers contract as the infection is brought under control. CD40$^{-/-}$ mice that survived the crisis point at 4 weeks postinfection had WT numbers of T cells at 5 weeks postinfection.

To establish whether CD40 ligation was involved in the induction of the Th1 response, the frequency of IFN-γ-producing T cells in the lymph nodes and the lungs of infected CD40$^{-/-}$ and WT mice was determined by ELISPOT assay. In WT mice, IFN-γ-producing T cells were detected in the lymph nodes and lungs as early as 2 weeks postinfection (Figures 4B and 4C). The numbers of IFN-γ-producing T cells increased in the lungs of WT mice up to 4 weeks postinfection, when the bacterial numbers stabilized (Figure 4C). In contrast, CD40$^{-/-}$ mice suffered from a major defect in the priming of IFN-γ-secreting T cells in the lymph nodes, which was most obvious at the peak priming point of 2 weeks postinfection (Figures 4B and 4C). This deficit in priming resulted in overall weaker IFN-γ responses in the lungs of CD40$^{-/-}$ mice, which finally reached WT levels at 5 weeks postinfection. Both CD4 and CD8 T cell responses were impaired; however, the absence of CD40 had a greater impact on CD4 T cells (Figure 4D). These results were confirmed by IFN-γ intracellular cytokine staining of T cells from the lungs of WT and CD40$^{-/-}$ mice at each time point (data not shown).

M. tuberculosis-infected CD40$^{-/-}$ and WT DCs were equally capable of inducing T cells from infected mice to produce IFN-γ upon in vitro restimulation (data not shown), indicating that T cells that had infiltrated the lungs of CD40$^{-/-}$ mice function independently of CD40/CD40L interaction (data not shown). These results suggest that CD40 is critical for the initiation of T cell responses within the secondary lymphoid organ, but not at the effector stage of T cell function within the lung following *M. tuberculosis* infection.

CD40$^{-/-}$ Mice that Survive the Crisis Point Succeed in Inducing IFN-γ Responses

The finding that some CD40$^{-/-}$ mice survived the crisis point and could live up to 5 months postinfection albeit with a higher bacterial burden was intriguing. To investigate the differences between resistant and susceptible CD40$^{-/-}$ mice, the cytokine profile in the lungs was analyzed by RPA. At 4 weeks postinfection, during the crisis point, we extracted RNA from the lungs of four moribund CD40$^{-/-}$ mice (Figure 5A, lanes 2, 4, 14, and 16), one CD40$^{-/-}$ mouse that was controlling infection (Figure 5A, lane 6), and three WT mice (Figure 5A, lanes 8, 10, and 12). CD40$^{-/-}$ mice that failed to control *M. tuberculosis* infection had ~1000-fold higher cfu in the lungs compared to WT mice. These mice had higher levels of IL-10 and IL-6 and no detectable IL-15 and IFN-γ mRNA (Figure 5A, lanes 2, 4, 14, and 16). In contrast, the relatively healthy CD40$^{-/-}$ mouse had ~10-fold more cfu in the lungs compared to WT mice, no IL-10 mRNA expression, and normal levels of IFN-γ and IL-15 (Figure 5A, compare lane 6 to lanes 8, 10, and 12). In the healthy CD40$^{-/-}$ mouse, a high level of IL-6 mRNA was also detected, indicating that irrespective of the disease status, the inflammation in the lungs of CD40$^{-/-}$ mice was significant (Figure 5A, compare lane 6 to lanes 8, 10, and 12).

As a group, CD40$^{-/-}$ mice had significantly reduced levels of IFN-γ mRNA over the course of infection (Figure 5B) and higher levels of IL-10 at 4 weeks postinfection (Figure 5C). Expression of IL-10 and IL-6 is likely due to the inflammation and extensive necrosis in the lungs of CD40$^{-/-}$ mice at 3 and 4 weeks postinfection. CD40$^{-/-}$ mice also had significantly less IL-12 mRNA in the lungs throughout the infection as shown by quantitative real-time RT-PCR (data not shown). No IL-4 mRNA was detected (data not shown).

These results confirmed that the susceptibility of CD40$^{-/-}$ mice to progressive *M. tuberculosis* infection was associated with a defect in the ability of these mice to produce IFN-γ in the lungs during acute infection. Those mice that survived were eventually able to recruit IFN-γ-producing T cells to the lungs.

Infection of CD40$^{-/-}$ Mice with Higher Inoculum Rescued CD40$^{-/-}$ Mice from Death

The duration and strength of TCR-mediated signals may dictate the level of costimulation required to achieve T cell activation (Bluestone, 1995; Mackey et al., 1998). Therefore, the antigen dose or number of priming events

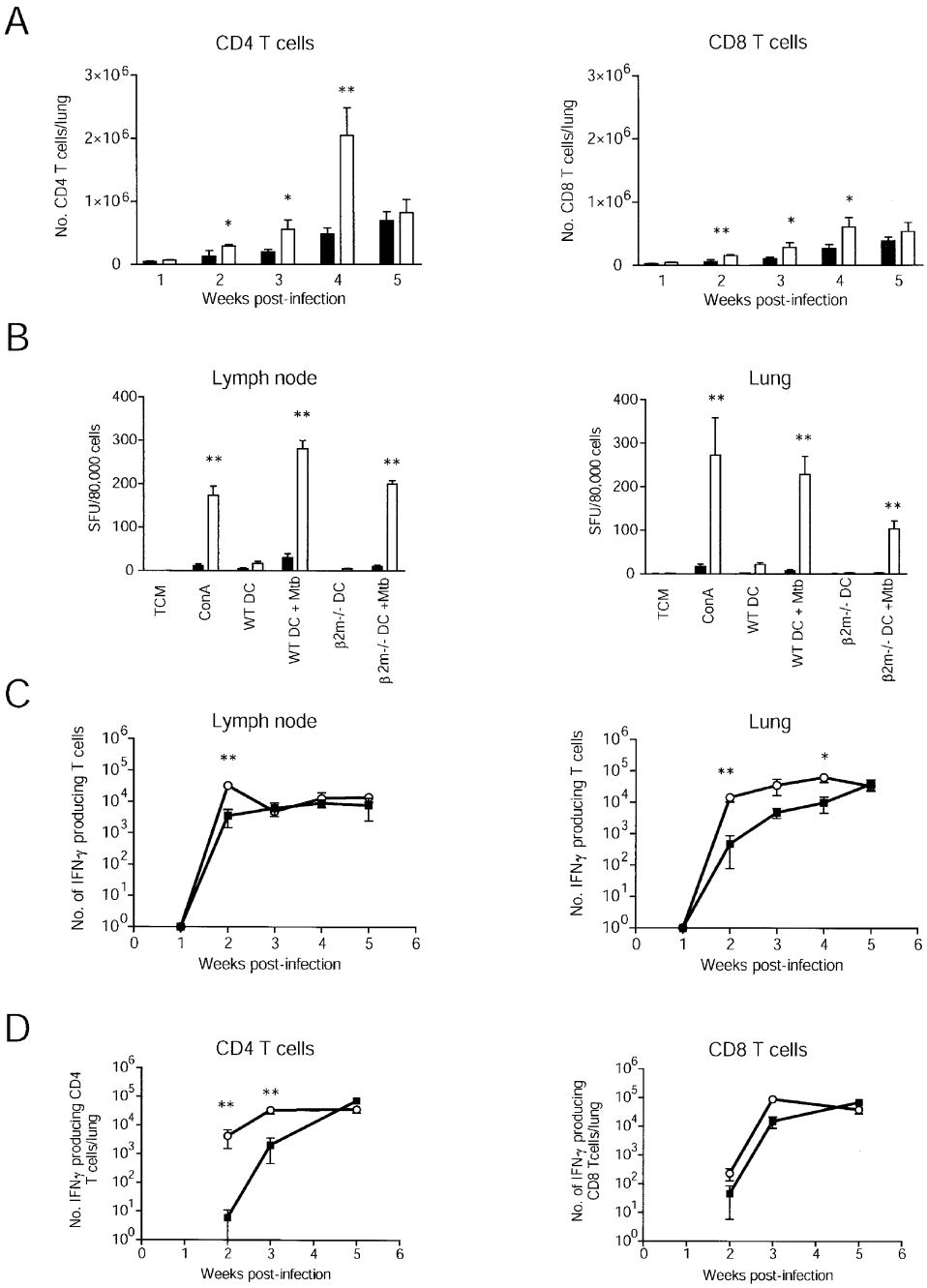

Figure 4. CD40$^{-/-}$ Mice Show Delayed Priming of IFN-γ-Producing T Cells in the Lymph Nodes and Overall Weaker IFN-γ Responses at the Site of Infection

(A) Lung cells from CD40$^{-/-}$ (■) and WT (□) mice were stained with anti-CD4 and anti-CD8 antibodies. The data are representative of three experiments, and five to six mice per experimental group were used at each time point.

(B) The number of IFN-γ-producing T cells from the lymph nodes and lungs of 2 week-infected CD40$^{-/-}$ (■) and WT (□) mice was assessed by ELISPOT.

(C) The number of sfu per 150,000 of lymph node cells or 80,000 lung cells was used to calculate the frequency of IFN-γ-producing cells in response to *M. tuberculosis*-infected WT DCs. The number of total IFN-γ-secreting T cells per lymph node or lung was calculated using the following formula: number of IFN-γ-producing cells = frequency × total number of cells per lymph node or lung. In each case, the number of sfu in response to uninfected DCs was subtracted from the number of sfu in response to *M. tuberculosis*-infected DCs before the calculations were made. ■ represents CD40$^{-/-}$, and ○ represents WT mice.

(D) Eighty thousand lung cells from CD40$^{-/-}$ (■) and WT (○) mice were incubated with either uninfected and *M. tuberculosis*-infected β2m$^{-/-}$ DCs or uninfected and *M. tuberculosis*-infected MHC II$^{-/-}$ DCs to estimate the number of IFN-γ-producing CD4 and CD8 T cells, respectively.

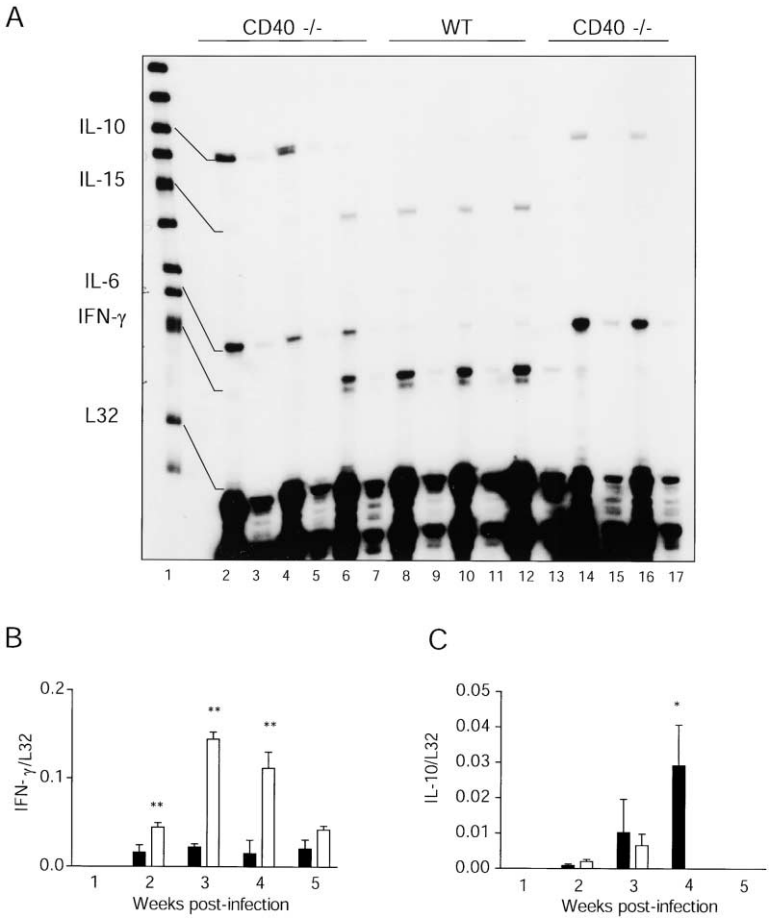

Figure 5. Cytokine Profile of Moribund CD40$^{-/-}$ Mice Showed Impaired Induction of IFN-γ Gene Expression

(A) At 4 weeks postinfection RNA was extracted from the lungs of four moribund CD40$^{-/-}$ mice (lanes 2, 4, 14, and 16), one CD40$^{-/-}$ mouse that controlled infection (lane 6), and three WT mice (lanes 8, 10, and 12) and analyzed by RPA. Lane 1 contains unprotected probe (marker), and each lane represents protected probe from an individual mouse or its 1:10 dilution.

(B and C) mRNA levels for IFN-γ and IL-10 were determined by RPA at weekly time points from the lungs of infected CD40$^{-/-}$ (■) and WT (□) mice. The results represent the mean ratio of the gene of interest and L32 housekeeping gene for four mice per experimental group per time point. The data are representative of two experiments.

may determine the requirement for CD40/CD40L interaction in the induction of *M. tuberculosis*-specific T cell responses. To address this question, CD40$^{-/-}$ mice were infected with 2×10^5 cfu/mouse intravenously (i.v.). CD40$^{-/-}$ mice had an approximately 10-fold higher number of cfu in the lungs 3 weeks after i.v. infection with *M. tuberculosis* (Figure 6A). In contrast to aerosol-infected CD40$^{-/-}$ mice in which the number of cfu continued to rise to high levels, CD40$^{-/-}$ mice maintained slightly higher but stable bacterial numbers after i.v. infection (Figure 6A). None of the CD40$^{-/-}$ mice died after i.v. *M. tuberculosis* infection during the study. Except for the 2-week time point, the overall numbers of CD4 and CD8 T cells in the lungs of CD40$^{-/-}$ mice were comparable to those in WT mice (Figure 6B). Normal priming in the lymph nodes and only slightly lower IFN-γ responses were observed in the lungs of CD40$^{-/-}$ mice at 2 weeks postinfection (Figure 6C). No difference in the number of IFN-γ-producing T cells was detected in the spleens of CD40$^{-/-}$ and WT mice following i.v. infection (Figure 6C, spleen). After low-dose aerosol challenge, there were on average 500 and 5000 IFN-γ-producing T cells in the lungs of CD40$^{-/-}$ mice at 2 and 3 weeks, respectively (Figure 4C, lung). After higher antigenic challenge a 17-fold and 2-fold increase in the number of IFN-γ-producing T cells was detected in the lungs of CD40$^{-/-}$mice at 2 and 3 weeks, respectively (Figure 6C, lung).

Aerosol infection with a higher inoculum (2- to 3-fold higher, ~100 cfu) was performed to address whether higher antigen load or systemic infection increased resistance of CD40$^{-/-}$ mice to *M. tuberculosis*. Similar to intravenous infection, CD40$^{-/-}$ mice had stable bacterial numbers, and none of the CD40$^{-/-}$ mice died after higher-dose aerosol infection. When compared to low-dose infection, the number of IFN-γ-producing T cells was 7-fold higher in the lymph nodes of CD40$^{-/-}$ mice at 3 weeks after high-dose infection, leading to 8.6- and 8-fold higher numbers of IFN-γ-producing T cells in the lungs of CD40$^{-/-}$ mice at 4 and 5 weeks, respectively (data not shown).

Thus, the markedly improved survival of CD40$^{-/-}$ mice is attributed to the induction of a stronger Th1 response characterized by a potent IFN-γ production in the lungs, solely as a result of higher antigen dose.

The Outcome of *M. tuberculosis* Infection in CD40$^{-/-}$ and CD40L$^{-/-}$ Mice Is Different

The finding that IFN-γ responses were impaired in CD40$^{-/-}$ mice after aerosol challenge was surprising, as a previous study reported that CD40L$^{-/-}$ mice developed mycobacteria-specific Th1 responses in the spleen and were resistant to intravenous infection with *M. tuberculosis* (Campos-Neto et al., 1998). This observation prompted us to investigate whether different routes of infection could influence the outcome of infection in CD40L$^{-/-}$ mice. In contrast to CD40$^{-/-}$ mice,

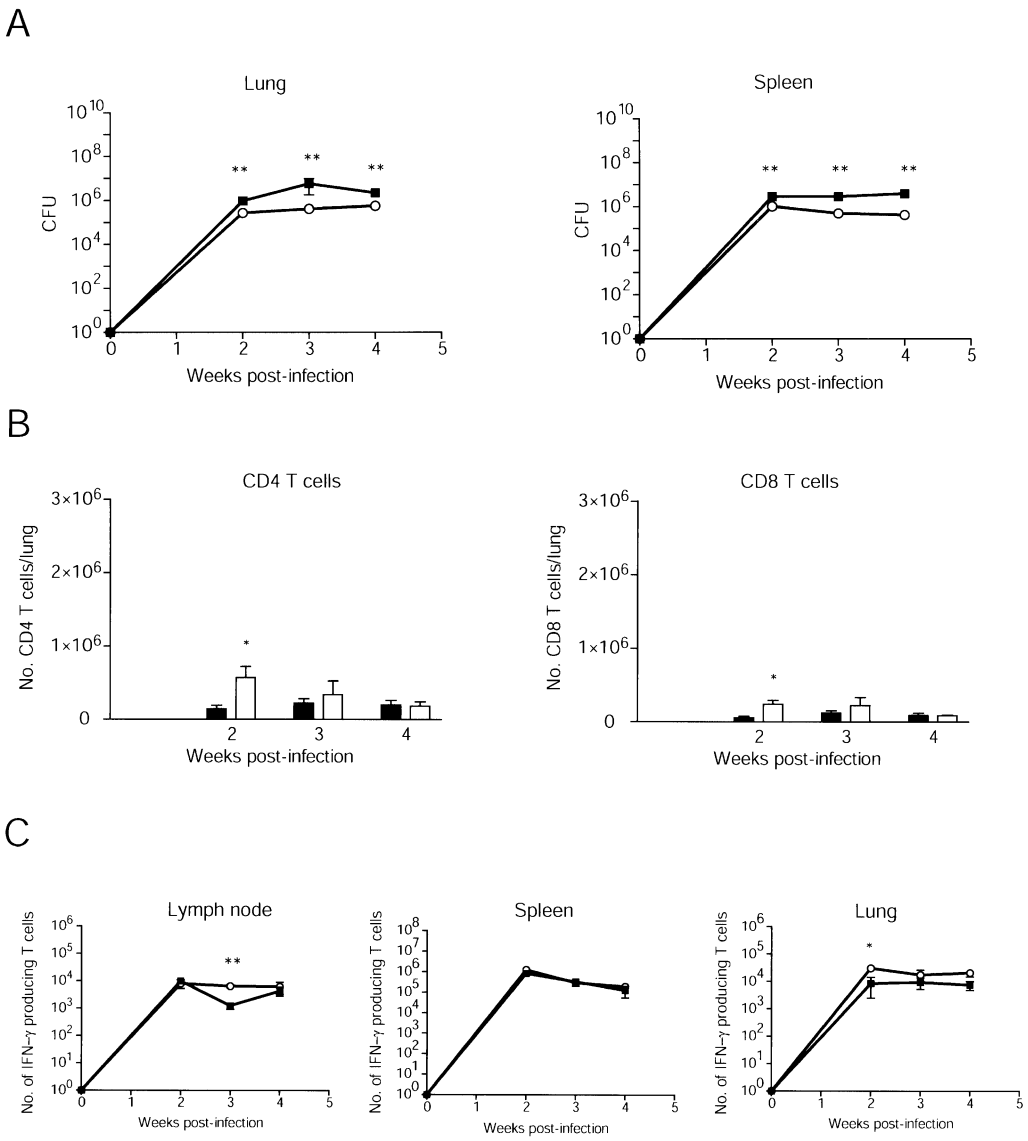

Figure 6. Systemic Infection of CD40$^{-/-}$ Mice with Higher Bacterial Inoculum Rescues CD40$^{-/-}$ Mice from Death

(A) CD40$^{-/-}$ (■) and WT (○) mice were infected with 2 × 10^5 *M. tuberculosis* bacilli intravenously. At weekly time points, serial dilutions of lung and spleen homogenates were plated for cfu determination.

(B) The CD4 and CD8 T cell responses in the lungs of CD40$^{-/-}$ (■) and WT (□) mice were analyzed using flow cytometry.

(C) 150,000 lymph node cells/well, 200,000 splenocytes/well, and 80,000 lung cells/well from CD40$^{-/-}$ (■) and WT (○) mice were incubated with uninfected or *M. tuberculosis*-infected WT DCs for 40 hr in an ELISPOT assay. The number of IFN-γ-producing T cells was calculated as follows: frequency × total number of cells per lymph node, spleen, or lung.

CD40L$^{-/-}$ or anti-CD40L-antibody-treated mice were resistant to low-dose (30–50 cfu) aerosol challenge with *M. tuberculosis* and controlled mycobacterial growth in the lungs and spleen comparably to control mice (Figures 7A and 7B and data not shown).

The magnitude of CD4 and CD8 T cell responses in the lungs of CD40L$^{-/-}$ and anti-CD40L-antibody-treated mice was similar to control mice, indicating that the development of protective T cell responses occurs normally in the absence of CD40L (Figure 7C and data not shown). As early as 2 weeks postinfection, there was a substantial number of IFN-γ-secreting T cells in the lungs and lymph nodes of CD40L$^{-/-}$ mice (Figure 7D). These results suggest that irrespective of the route of infection CD40L$^{-/-}$ mice are resistant to *M. tuberculosis* challenge due to their ability to prime mycobacteria-specific Th1 cells that migrate to the lungs early in infection, before the crisis point that we saw in CD40$^{-/-}$ mice. The results obtained in mice treated with blocking anti-CD40L antibody correlated well with the results using CD40L$^{-/-}$ mice, confirming that resistance in CD40L$^{-/-}$ mice was not due to compensatory mechanisms in a knockout strain.

Discussion

In this study we demonstrate that CD40 is essential for the induction of protective immunity against aerosol

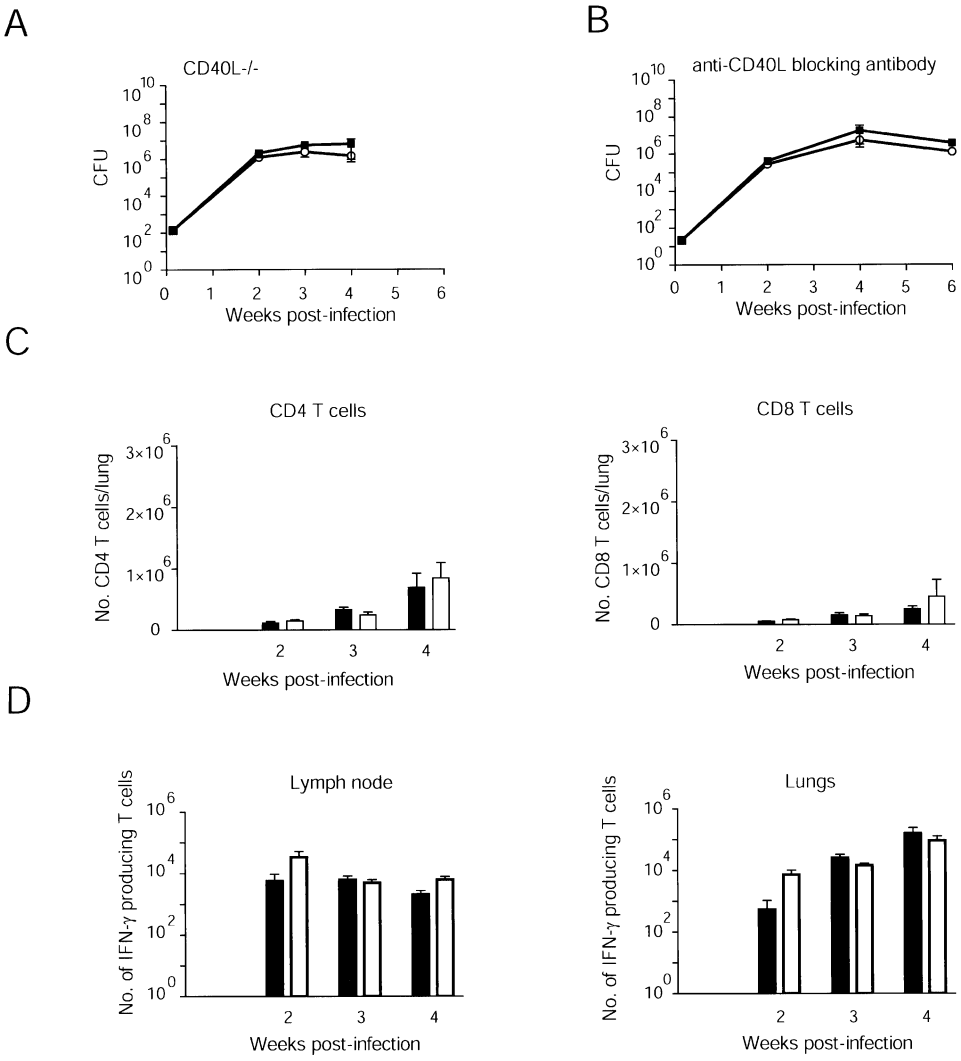

Figure 7. CD40L$^{-/-}$ Mice Are Not Susceptible to Aerosol *M. tuberculosis* Infection

(A and B) Mice were infected with 30–50 cfu of *M. tuberculosis* via aerosol. Serial dilutions of lung homogenates from CD40L$^{-/-}$ mice (■, [A]) or anti-CD40L blocking antibody-treated WT mice (■; [B]) and their respective controls (○) were plated for cfu determination.

(C) The number of CD4 and CD8 T cells in the lungs of CD40L$^{-/-}$ (■) and WT (□) mice was determined by staining lung single-cell suspensions with anti-CD4 and anti-CD8 antibodies, respectively.

(D) The number of IFN-γ-producing T cells per lymph node or lung from CD40L$^{-/-}$ (■) and WT mice (□) was estimated by ELISPOT. The number of IFN-γ-producing T cells was calculated as described in the legends to Figures 4 and 6. The results represent the mean of three to four mice per time point, and error bars are standard error of mean. Statistical significance was determined by the Student's t test.

challenge with *M. tuberculosis*. The susceptibility of CD40$^{-/-}$ mice to *M. tuberculosis* infection was attributed to the failure of these mice to rapidly mount protective Th1 responses. The underlying cause for poor induction of the Th1 response was due to deficient in vivo IL-12 production by CD40$^{-/-}$ DCs upon *M. tuberculosis* infection. Interestingly, the outcome of infection in CD40$^{-/-}$ and CD40L$^{-/-}$ mice was markedly different. This dichotomy implies that CD40 ligation, but not CD40L, is essential for the induction of a robust Th1 response in *M. tuberculosis* infection. In the absence of CD40L, stimulation through CD40 could be achieved by an alternative ligand. Here, we demonstrate that infection with *M. tuberculosis* or treatment with *M. tuberculosis* Hsp70 stimulates IL-12 production from WT DCs in a CD40-dependent manner.

At 4 weeks postinfection, nearly 40% of CD40$^{-/-}$ mice died, and histological analysis revealed excessive necrosis and consolidation of airspaces leading to hypoxia and respiratory distress in susceptible mice. However, some CD40$^{-/-}$ mice lived for up to 5 months postinfection albeit with higher bacterial numbers in the lungs when compared with WT mice. This intriguing finding prompted us to determine the cause of susceptibility in CD40$^{-/-}$ mice and examine why a subset of CD40$^{-/-}$ mice were capable of controlling *M. tuberculosis* infection.

Our results indicate that the susceptibility of CD40$^{-/-}$ mice to *M. tuberculosis* infection stems from their inability to produce IFN-γ early in infection. This impaired Th1 response is related to the inefficient priming of naive T cells by CD40$^{-/-}$ DCs compared to WT DCs. It is generally believed that infection of DCs with *M. tuberculosis* is sufficient to mature them into potent antigen-

presenting cells. Infection of DCs with *M. tuberculosis* results in upregulation of antigen-presenting, costimulatory, and adhesion molecules in the production of inflammatory cytokines such as IL-1, IL-12, and TNF-α (Bodnar et al., 2001; Gonzalez-Juarrero and Orme, 2001; Henderson et al., 1997), and efficient in vitro priming of *M. tuberculosis*-specific CTL responses (Serbina et al., 2001). Despite the fact that *M. tuberculosis*-infected CD40$^{-/-}$ DCs displayed phenotypic changes indicative of maturation, such as cell surface marker upregulation, their ability to prime naive T cells was diminished compared to WT DCs. *M. tuberculosis*-infected WT DCs produced greater than four times more IL-12 than infected CD40$^{-/-}$ DCs. Furthermore, attenuated in vivo IL-12 production may have a direct effect on the priming efficiency of Th1 responses in the lymph nodes of CD40$^{-/-}$ mice. Addition of IL-12 to CD40$^{-/-}$ DC-T cell cocultures enhanced the priming of potent IFN-γ T cell responses.

Our results indicate that CD40$^{-/-}$ mice manifested a major defect in the in vivo priming of *M. tuberculosis*-specific, IFN-γ T cell responses. This early defect in IFN-γ production in the lymph nodes of CD40$^{-/-}$ mice had a significant impact on the magnitude of IFN-γ responses in the lungs. Infiltration of IFN-γ-producing T cells into the lungs of CD40$^{-/-}$ mice was substantially delayed compared with WT mice. While WT mice responded early to *M. tuberculosis* challenge with IFN-γ production, poor priming in CD40$^{-/-}$ mice provided a window of opportunity for *M. tuberculosis* to overwhelm the immune system of CD40$^{-/-}$ mice. These results underscore the importance of early IFN-γ production in resistance to *M. tuberculosis* infection as previously suggested with CD4 T cell-deficient mice (Caruso et al., 1999). *M. tuberculosis*-infected CD40$^{-/-}$ DCs could restimulate in vivo primed T cells to produce IFN-γ, suggesting that the defect in CD40$^{-/-}$ mice is at the level of priming and that CD40 is not required for the effector function of T cells.

It has been proposed that the strength of signal and duration of TCR stimulation can determine the level of costimulation required to achieve efficient T cell activation (Bluestone, 1995; Mackey et al., 1998). High antigen dose and prolonged TCR ligation were shown to overcome the need for costimulation in the induction of T cell responses (Kundig et al., 1996). CD40$^{-/-}$ mice did not exhibit a permanent impairment but rather a 4 week delay in IFN-γ production in the lungs. This suggests that continuous replication of mycobacteria within the lungs of CD40$^{-/-}$ mice may generate a sufficient antigen dose that will elicit repeated and prolonged TCR stimulation, thereby overcoming the need for CD40/CD40L interaction as proposed for some viral infections (reviewed in Mackey et al., 1998). Thus, those mice that survived the critical point may have received a slightly higher dose of bacteria during aerosol infection or supported a higher antigen load early in the lungs.

To evaluate the effect of antigen dose and systemic infection on CD40-dependent induction of Th1 responses, we infected CD40$^{-/-}$ mice with a higher bacterial inoculum intravenously and via aerosol. High-dose intravenous and aerosol infections resulted in improved induction of IFN-γ responses, maintenance of stable bacterial numbers in the lungs and spleens, and survival of mice throughout duration of the study. These results suggest that high antigen dose and priming at multiple sites achieved through systemic infection or immunization may overcome the need for CD40 ligation in the induction of mycobacteria-specific IFN-γ responses.

The susceptibility of CD40$^{-/-}$ mice to low-dose aerosol challenge was unexpected, as a previous study reported that CD40L$^{-/-}$ mice were resistant to intravenous *M. tuberculosis* infection (Campos-Neto et al., 1998). To exclude the possibility that the route of infection could have an effect on the outcome of infection, we infected CD40L$^{-/-}$ and WT mice that were treated with the blocking anti-CD40L antibody with a low dose of *M. tuberculosis* by aerosol. CD40L$^{-/-}$ mice were resistant to *M. tuberculosis* infection irrespective of the infection route. The development of Th1 responses in CD40L$^{-/-}$ and anti-CD40L antibody-treated mice was similar to that of control mice, indicating that the absence of CD40L has a negligible impact on the generation of IFN-γ responses in *M. tuberculosis* infection.

These data indicate that CD40, but not CD40L, is required for the optimal priming of T cells and control of *M. tuberculosis* infection. One possible explanation could be the existence of another host-derived ligand for CD40, as shown for other members of the TNF-R superfamily (Chalupny et al., 1992; Van Kooten and Banchereau, 1996). Alternatively, a *M. tuberculosis*-encoded protein may directly ligate CD40 on APCs, thereby modulating their function. Our results support that a *M. tuberculosis*-derived product induces IL-12 production by DCs through CD40 ligation. In the absence of exogenous IFN-γ or T cells, the level of IL-12 produced by *M. tuberculosis*-infected WT DCs was four times greater than the amount of IL-12 produced by *M. tuberculosis*-infected CD40$^{-/-}$ DCs. Furthermore, we found that recombinant *M. tuberculosis* Hsp70 stimulated significant IL-12 production by WT but not CD40$^{-/-}$ DCs, suggesting that Hsp70 is an alternative ligand for CD40. A recent publication reported that mycobacterial Hsp70 binds to CD40 and stimulates human mononuclear cells to release CC-chemokines RANTES, MIP-1α, and MIP-1β (Wang et al., 2001). Thus, our data and this work strongly support a role for mycobacterial-derived proteins in the stimulation of CD40 for priming immune responses.

Our initial interest in CD40/CD40L interaction stemmed from experiments in which CD4 T cell depletion in chronically infected mice resulted in the death of mice despite normal levels of IFN-γ and NOS2 expression in macrophages (Scanga et al., 2000). Since CD40L is primarily expressed by activated CD4 T cells, we sought to determine whether additional roles of CD4 T cells in the control of *M. tuberculosis* infection are mediated through CD40/CD40L interaction. Our results show that the phenotype of CD40$^{-/-}$ mice differs considerably from that of CD40L$^{-/-}$ and CD4$^{-/-}$ mice (data not shown), indicating that CD40/CD40L interaction is not the mechanism by which CD4 T cells contribute to the control of acute or chronic *M. tuberculosis* infection.

In conclusion, our results demonstrate the importance of CD40 in the generation of protective immunity against *M. tuberculosis*. The failure of CD40$^{-/-}$ mice to control *M. tuberculosis* infection is attributed to inefficient priming of IFN-γ T cell responses. CD40 dependence on induction of IFN-γ responses appears to be a function

of antigen dose as intravenous, and aerosol infection of CD40$^{-/-}$ mice with a higher dose of *M. tuberculosis* overcomes the need for costimulation in the induction of IFN-γ responses. These results may have important implications for vaccine development and priming of immune responses in humans following *M. tuberculosis* infection as the rate at which T cell responses are primed could influence progression to disease or containment of the infection.

Experimental Procedures

Mice

C57BL/6 mice, CD40L$^{-/-}$ (B6.129S2-*Tnfsf5*^{tm1Imx}) mice, and CD40$^{-/-}$ (B6.129P2-*Tnfrsf5*^{tm1Kik}) breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME). β2m$^{-/-}$, MHC II$^{-/-}$, and CD40$^{-/-}$ mice were bred in the University of Pittsburgh Biotechnology Center. All mice were maintained under specific pathogen-free conditions and used at 8–12 weeks of age. The University Institutional Animal Care and Use Committee approved all animal protocols employed in the study.

Bacteria and Infections

Aerosol and intravenous infections with *M. tuberculosis* (Erdman strain, Trudeau Institute, Saranac Lake, NY) were performed as described previously (Serbina et al., 2000). In the anti-CD40L blocking study, C57BL/6 mice were injected with 250 μg/injection of blocking anti-CD40L antibody MR1 (a generous gift from Dr. Robert Hendricks) prior to infection and every other day throughout the duration of the study.

Cfu Determination

Bacterial burden was determined by plating serial dilutions of lung and spleen homogenates onto 7H10 agar plates (Difco). Plates were incubated at 37°C in 5% CO_2 for 21 days prior to counting colonies.

Histology and Immunohistochemistry

For histological analysis, organs were fixed in 10% normal buffered formalin and embedded in paraffin, and 6 μm sections were stained with hematoxylin and eosin.

Flow Cytometry

Lung and lymph node single-cell suspensions were prepared and stained as described previously (Serbina et al., 2000). Cells were stained with anti-CD4 (clone H129.19), anti-CD8 (clone 53-6.7), anti-CD69 (clone H1.2F3), anti-CD40 (clone 3/23), anti-CD11c (clone HL3), anti-B220 (clone RA3-6B2), anti-MHC class I (H-2D^b; clone KH95), anti-MHC class II (I-A^b; clone AF6-120.1), and anti-B7.2 (clone GL1) fluorescently conjugated antibodies. All antibodies were purchased from BD Pharmingen (San Diego, CA). Cells were collected on a FACSCaliber (Beckon Dickinson) and analyzed by CellQuest software (Becton Dickinson, Immunocytometry Systems, San Jose, CA) or FlowJo (Tree Star Inc, San Carlos, CA).

Culture of Bone Marrow-Derived Macrophages and Dendritic Cells

Macrophages and dendritic cells were generated from bone marrow of C57BL/6, CD40$^{-/-}$, MHC II$^{-/-}$, or β2m$^{-/-}$ mice, as described previously (Serbina et al., 2000).

Macrophage Killing Assay and Nitrite Production

Bone marrow-derived macrophages were plated in U-bottom, 96-well plates (triplicates) at 2 $\times$ 10^5/well and infected with *M. tuberculosis* at MOI 1.5 overnight. The next day T cells were harvested from the lungs of 4 week-infected mice, and single-cell suspensions were divided into two samples. In unfractionated sample, macrophages were removed by incubating cell suspensions in NUNC LabTek plates at 37°C for 2 hr. In CD8-depleted sample, cells were incubated with 1:4 dilution of hybridoma 2.43 supernatant (anti-CD8) at 4°C for 30 min. Cells were washed once, and the cell pellet was suspended in T cell media (RPMI, 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 25 mM HEPES, 50 μM 2-ME [Sigma]). Cells were added to goat anti-rat IgG (Zymed Laboratories)-coated plates (10 μg/10 ml of 0.05M Tris buffer/plate, 4°C, O/N) and incubated at room temperature for 1 hr. Following incubation, nonadherent cells were removed, and plates were washed twice with PBS. Cells were suspended at 1 $\times$ 10^6/ml. Efficiency of CD8 T cell depletion was confirmed by flow cytometry. After overnight infection, macrophages were washed twice with warm DMEM, and 200 μl/well of T cell media, 250 U/ml IFN-γ + 3 μg/ml LPS, CD8-depleted lung cells (2 $\times$ 10^5/well) or unfractionated lung cells (2 $\times$ 10^5/well) were added to the wells. At this time point, macrophages were lysed in a subset of wells to determine the input number of intracellular bacteria.

Following 3 day incubation at 37°C, the number of intracellular bacteria was determined by lysing adherent macrophages with 1% saponin for 10 min. Cell lysates were collected and sonicated for 10 s, and serial dilutions were plated on 7H10 agar plates. Nitrite in the supernatants was measured by Greiss assay (Bodnar et al., 2001).

Functional Characterization of CD40$^{-/-}$ and WT Dendritic Cells

Uninfected and *M. tuberculosis*-infected WT DCs were treated with stimulating anti-CD40 antibody (BD Pharmingen; clone 3/23) or rIgG2a isotype control (BD Pharmingen; clone A110-2) at 3.5 μg/10^6 cells/ml on ice for 30 min. Following antibody treatment uninfected and *M. tuberculosis*-infected WT and CD40$^{-/-}$ DCs were seeded at 1 $\times$ 10^6/ml in 24-well plates in DC media supplemented with GM-CSF and IL-4 (1000 U/ml) for 24 hr. Supernatants were filter sterilized, and the amount of IL-12 was determined by ELISA. Dendritic cells were stained with anti-CD4, -CD8, -CD3, MHC class I, MHC class II, and B7.2 antibodies. Flow cytometric analysis revealed that dendritic cell preparations used for either *M. tuberculosis* infection or HSP70 ligation experiments did not contain CD3$^+$ T cells and that they expressed high levels of MHC class I, MHC class II, and B7.2. For Hsp70 binding experiments, uninfected WT and CD40$^{-/-}$ DCs were incubated with either 10 μg/ml or 1 μg/ml of purified, LPS-free Hsp70 (generously provided by Dr. John Belisle, the NIH Tuberculosis Reagents Contract NO1 AI-75320).

In Vitro Priming Assay

Single-cell suspensions from spleens of C57BL/6 mice, after lysis of red blood cells with NH_4-Tris solution at room temperature for 2 min, were treated with anti-B220 antibody (BD Pharmingen; clone RA3-6B2) at 10 μg/10^7 cells/ml at 4°C for 30 min, and the cells were added to goat anti-rat IgG-coated plates (Zymed Laboratories) for 1 hr at room temperature. Nonadherent cells were collected and efficiency of B cell depletion was confirmed by flow cytometry. The remaining splenocytes were cultured in T cell media supplemented with 20 U/ml of IL-2 (Roche) with either *M. tuberculosis*-infected CD40$^{-/-}$ or WT DCs at 1:10 (DC:T cell) ratio for 7 days. In IL-12 rescue assay, recombinant murine IL-12 (a generous gift from Genetics Institute, Cambridge, MA) was added to CD40$^{-/-}$ DC-T cell cocultures at 50 ng/ml. Midway through T cell-DC coculture, 100 μl of supernatants was removed from the wells, and the amount of IL-12 was measured using ELISA. Fresh T cell media (100 μl) supplemented with IL-2 (20 U/ml) was added for an additional 4 days of culture. After the 7 day incubation, cells were used in an IFN-γ ELISPOT to determine the frequency of IFN-γ-producing cells in response to T cell media (negative control), ConA (positive control; 10 μg/well [Sigma]), uninfected, and *M. tuberculosis*-infected DCs (1:2, DC:T cell ratio).

ELISA

IL-12 was quantitated using ELISA as described previously (Bodnar et al., 2001). The capture anti-IL12 antibody (Biosource International; clone C15.6) was used at 4 μg/ml in the binding buffer (0.1 M Na_2HPO_4 [pH 9.0]) overnight at 4°C. The biotinylated anti-IL12 antibody (Biosource International; clone 17.15), which detects both p40/p70 heterodimer, was added at 2 μg/ml in the incubation buffer for 1 hr at room temperature. Functional IL-12 p70 was detected using Quantikine M immunoassay kit (R&D Systems, Minneapolis, MN).

ELISPOT

Millipore Multiscreen 96-well MAIPS4510 plates (Millipore Corp, Bedford, MA) were coated with capture anti-IFN-γ antibody (BD

Pharmingen; clone R4-6A2) in PBS at 10 μg/ml overnight at 4°C. The next day, the plates were washed with PBS and blocked with RPMI/15% FBS for 1–2 hr at room temperature. Lung and lymph node single-cell suspensions were prepared as described previously (Serbina et al., 2000) and plated at 80,000 lung cells/well or 150,000 lymph node cells/well in T cell media supplemented with IL-2 at a final concentration of 20 U/ml. Lung and lymph node cells were either cultured in T cell media alone, ConA (10 μg/well [Sigma]), uninfected, and *M. tuberculosis*-infected WT DCs (total IFN-γ production), MHC $II^{-/-}$ DCs (IFN-γ production by CD8 T cells), and $\beta 2m^{-/-}$ DCs (IFN-γ production by CD4 T cells) at 1:2 (DC:T cell) ratio at 37°C for ~40 hr. Following incubation, plates were washed with PBS/0.1% Tween 20 and biotinylated anti-IFN-γ antibody (BD Pharmingen; clone XMG 1.2) was added at 5 μg/ml in PBS/0.5%BSA/0.1% Tween 20 at 37°C for 2 hr. Avidin Peroxidase Complex (PK-6100; Vector Laboratories) was prepared as directed by the manufacturer and added to the plates at 100 μl/well for 1 hr at room temperature. Following incubation, plates were washed with PBS/0.1% Tween 20 and developed by adding Vectastain AEC substrate (SK-4200; Vector Laboratories) prepared according to the manufacturer's instructions. The spot forming units (sfu) per well were counted using ELISpot reader. The cut-off number of sfu accurately measured by the ELISpot reader is 1500 sfu/well. In IL-12 rescue assay, the wells that were too numerous to count (TNTC) or were solid red were estimated to be >1500 sfu/well.

Quantitative RT-PCR

Total lung and lymph node RNA was extracted using Trizol (Life Technology, Green Island, NY) and RNA extraction kit as directed by the manufacturer (Qiagen, Valencia, CA). cDNA synthesis was performed using the Superscript II enzyme system according to the manufacturer's instructions (Quiagen). We adopted a relative gene expression method as described previously (Liu and Saint, 2002). In our assay, we used RNA isolated from the lungs of uninfected mice as a calibrator, and we used HPRT as a normalizer gene. Relative gene expression was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct$ (gene of interest) − Ct (normalizer) and the $\Delta\Delta Ct = \Delta Ct$ (sample) − ΔCt (calibrator). We used published sequences for the IL-12 primer and probe sets (Giulietti et al., 2001) at 400 nM and 250 nM concentrations, respectively.

RNase Protection Assay

A custom-made template set mck-2b (NOS2, IL-4, IL-12p40, TNF-α, IL-1β, IL-1α, IFN-γ), template set mck-1 (IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, IFN-γ), and multiprobe RNase protection assay (RPA) system (BD Pharmingen) were used to determine mRNA levels for genes of interest at designated time points. The relative gene expression was quantified by densitometer (ImageQuant Software, Molecular Dynamics, Sunnyvale, CA) and compared to the abundance of the housekeeping gene, L32.

Statistics

The results represent the mean ± standard error of mean. Statistical significance was calculated using the Student's t test. For comparison of cfu between $CD40^{-/-}$ and WT mice, the raw data were transformed into log numbers prior to statistical analysis. *p value ≤ 0.05; **p value ≤ 0.01.

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